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Solid Phase Synthesis Of 5-Hydroxymethyluracil Containing DNA

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Abstract

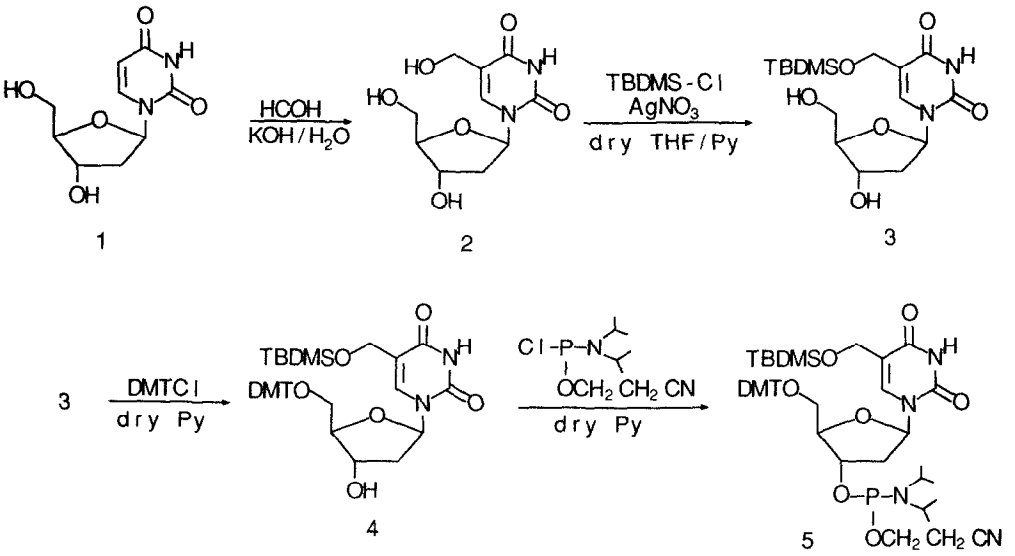
The synthesis of 3'-O-(diisopropylamino-2-cyanoethoxyphosphinyl)-5'-O-(4,4'-dimethoxytrityl)-5-(*tert*-butyldimethylsiloxymethyl)-2'-deoxyuridine (**5**) and its utilization for the preparation of 5-hydroxymethyluracil (hmU) containing oligodeoxyribonucleotides by means of automated synthesis are described.

The presence of 5-hydroxymethyl-2'-deoxyuridine (hmU) in DNA is of great biological importance on a number of levels. Certain bacteriophages such as SPO1 contain hmU DNA as well as hmU specific binding proteins[1,2]. It has also been known that hmU is produced in natural DNA by an oxidative attack on the methyl group of thymine or, alternatively, *via* the deamination of 5-hydroxymethylcytosine which can be formed as a product of the oxidation of 5-methylcytosine in a reaction identical to that which yields hmU from uracil. Therefore it is believed that 5-hydroxymethyluracil could be a mutagenic lesion[3]. Recently, the production of hmU containing DNA has been linked to high fat diets[4], and the nucleoside has been shown to be a promising anti-leukemic and antiviral compound, alone or in combination with other drugs[5-7].

To the best of our knowledge, the preparation of a suitably protected hmU monomer for incorporation into synthetic oligodeoxyribonucleotides *via* standard phosphoroamidite methods has not been reported. The ability to produce large amounts of hmU containing DNA of defined sequences would be valuable to advancing the understanding of its biological function and chemistry. Moreover, these oligonucleotides will allow the physico-chemical studies of the effects of the substitution of thymine with hmU on the stability of the double helix[8]. For these reasons we have prepared the derivative **5** which can be used as a standard precursor in routine DNA syntheses. The correct incorporation of compound **5** into DNA oligomers was checked by the synthesis of several hmU oligomers including the hmU containing analogue of the well studied Dickerson dodecamer[9] CGCGAAhmUhmUCGCG.

The synthesis of the fully protected hmU (**5**) was carried out through the reactions outlined in the scheme. 5-Hydroxymethyl-2'-deoxyuridine (**2**) was prepared by hydroxymethylation of 2'-deoxyuridine (**1**) with paraformaldehyde in a KOH solution, according to the reported procedure[10] with minor modifications, and purified by MPLC on silica gel by eluting with increasing amounts of methanol in chloroform (from 5 to 30 %).

SCHEME



The presence of an additional hydroxyl functionality required a selective protection compatible with an acid-labile protecting group, such as the standard 4,4'-dimethoxytrityl (DMT) group, that would not be removed when deprotecting the 5' hydroxyl group for nucleotide addition during the oligo synthesis. For this purpose we used the *tert*-butyldimethylsilyl (TBDMS) ether, a protecting group which has greatly facilitated the synthesis of oligoribonucleotides[11]. Thus, hmU (**2**, 1 mmole) was dissolved in dry pyridine and dry THF (1:2) and silver nitrate was added (1.5 mmole) with stirring until it had completely dissolved[12]. Then, *tert*-butyldimethylsilyl chloride (TBDMS-Cl, 1.5 mmole) was added and the resulting mixture was stirred at room temperature for two hours. After filtration and addition of water the product was extracted into chloroform. Flash chromatography of the product on silica gel column (eluent: from chloroform to chloroform/methanol 8:2) yielded, along with small amounts of the 5,5'-disilylated derivative, a mixture (85:15)[13] of **3** and its 5'-silylated regioisomer[14]. This mixture was resolved into the individual components by HPLC (Lichrosorb Si60, silica gel column, 7 μm, 250-25, Merck; eluent: ethyl acetate/methanol 98:2) thus yielding pure **3**[15]. Compound **3** was converted to the DMT derivative **4**[16] which, in turn, gave the desired phosphoramidite **5**[17] using the standard procedures[18].

Compound **5** was used for the preparation of hmU containing DNA oligomers following the usual protocols[19]. During the automated syntheses, compound **5** exhibited similar coupling efficiences as the commercially available phosphoroamidites derived from the normal DNA nucleosides. Final ammonia treatment partially removed the TBDMS protecting groups and complete deprotection was achieved by a one hour treatment with 80% acetic acid at room temperature. Purification of the oligonucleotides was carried out by ion exchange HPLC on a Partisil 10 SAX column using linear gradient of KH_2PO_4, 30% CH_3CN, pH=7 from 10 mM to 0.35 M followed by gel filtration on a Biogel P2 column (eluent H_2O/EtOH 8:2). Enzymatic analises on the synthesized oligomers with snake venom phosphodiesterase confirmed the exclusive existence of 5'-3' linkages.

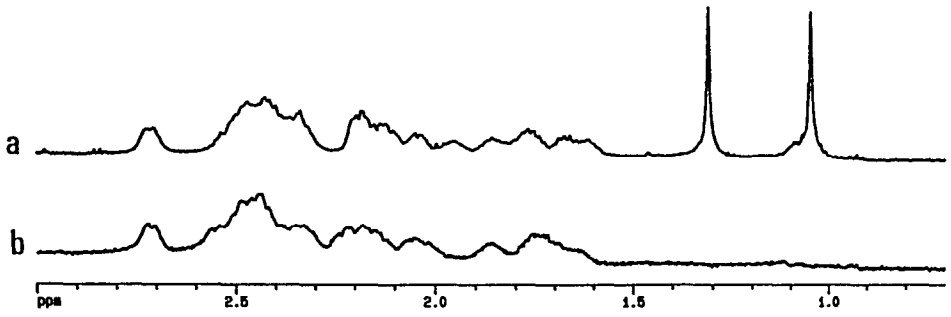


Fig 1. Aliphatic region of the 500 MHz ^{1}H-NMR spectra of the Dickerson dodecamer (a) and its hmU containing analogue (b).

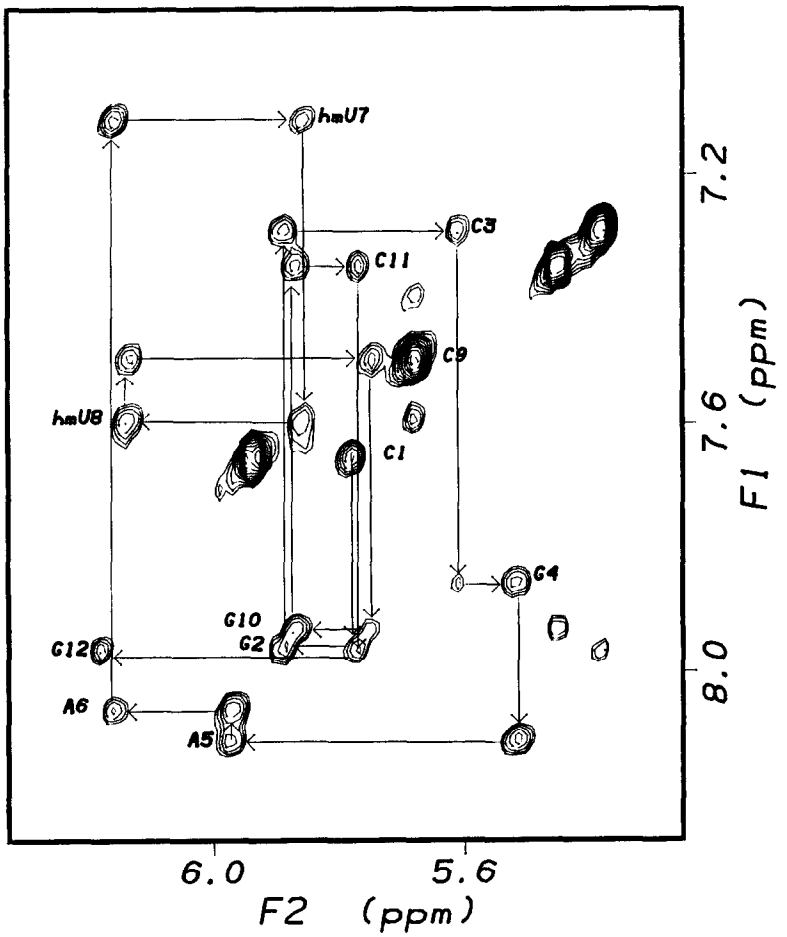


Fig.2. The 1' to base region of the phase sensitive NOESY of the hmU dodecamer CGCGAAhmUhmUCGCG

Replacement of thymines with hmU results in NMR spectra which lack the upfield methyl resonances as shown by fig. 1 where the aliphatic regions of the ^{1}H-NMR spectra of the Dickerson dodecamer (a) and its hmU analogue (b) are reported. In the hmU containing dodecamer, the methylene protons of the hmU bases appear between 3.4 and 4.1 ppm and are unresolved in the one-dimensional spectra due to overlapping 4',5' and 5'' sugar protons. The 1' sugar proton to base proton region of a phase sensitive NOESY[20] spectrum of the hmU containing dodecamer is shown in fig. 2. Standard sequential assignment strategies[21] were employed to verify the correctness of the desired sequence. Inter- and intra-nucleotide NOE's involving the methylene protons of the hmU bases were also observed (data not shown), thus further confirming the successful synthesis of the modified dodecamer. Further studies concerning the structures and properties of this oligomer are currently in progress and will be published elsewhere.

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References

1. Johnson, G.G.; Geiduscheck, E.P. *Biochemistry* **1977**, *16*, 1473-85.
2. Greene, J.R.; Geiduscheck, E.P. *EMBO J.* **1985**, *4*, 1345-49.
3. Levy, D.D.; Teebor, G.W. *Nucleic Acids Research* **1991**, *19*, 3337-43 and references cited therein.
4. Djuric, Z.; Heilbrun, L.K.; Reading, B.A.; Boomer, A.; Valeriote, F.A.; Martino, S. *J. National Cancer Inst.* **1991**, *83*, 766-69.
5. Vilpo, J.A.; Vilpo, L.M. *Cancer Res.* **1988**, *48*, 3117-22.
6. Vilpo, J.A.; Suvanto, E.; Kangas, L. *Leuk. Res.* **1987**, *11*, 877-80.
7. De Clercq, E.; Beres, J.; Bentrude, W.G. *Mol. Pharmacol.* **1987**, *32*, 286-92.
8. Kallen, R.G.; Simon, M.; Marmour, J. *J. Mol. Biol.* **1962**, *5*, 248-50.
9. Nerdal, W.; Hare, D.R.; Reid, B.R. *Biochemistry* **1989**, *28*, 10008-21 and references cited therein.
10. Shiau, G.T.; Schinazi, R.F.; Chen, M.S.; Prusoff, W.H. *J Med. Chem.* **1980**, *23*, 127-33.
11. Usman, N.; Ogilvie, K.K.; Jiang, M.Y.; Cedergren, R.J. *J. Am. Soc.* **1987**, *109*, 7845-54.
12. Hakimelahi, G.H.; Proba, Z.A.; Ogilvie, K.K. *Can. J. Chem.* **1982**, *60*, 1106-13.
13. The silylation in pyridine, without addition of silver nitrate, is slightly less selective, leading to a mixture 4:1 of the two regioisomers.
14. In order to avoid competition between the two primary hydroxyl groups towards TBDMS-Cl, we tried to introduce the 5-hydroxymethyl function in 5'-dimethoxytrityl-2'-deoxyuridine (DMT-dU). Due to the very scanty, if any, solubility of DMT-dU in the aqueous KOH solution, we tested the hydroxymethylation reaction in different solvent mixtures but our attempts were unsuccessful.
15. **3**. 65% yield. ^{1}H-NMR (250 MHz, $CDCl_3$, assignment confirmed by spin decoupling experiments): δ 7.50 (1H, bs, 6-H); 6.19 (1H, t J=6.8 Hz, 1'-H); 4.59 (1H, m, 3'-H); 4.49 (2H, bs, 5-CH_2-O); 4.02 (1H, q, J=3.7 Hz, 4'-H); 3.85 (2H, further coupled AB system, 5'-H_2); 2.45 (1H, m, 2'-H); 2.30 (2H, m, 2''-H and 5'-OH); 1.97 (1H, d J=3.7 Hz, 3'-OH); 0.93 (9H, s, $(CH_3)_3$C-Si) and 0.11 (6H, s, $(CH_3)_2$Si).
^{13}C-NMR (125 MHz, CD_3OD): δ 164.43 (C=O,C4); 151.85 (C=O, C2); 138.42 (CH,C6); 114.91 (C,C5); 88.49 (CH,C4'); 86.04 (CH,C1'), 72.10 (CH,C3'); 62.74 (CH_2,C5'); 59.04 (CH_2,5-$\underline{C}H_2$-O-Si); 40.80 (CH_2,C2'); 26.11 (CH_3,($\underline{C}H_3)_3$C-Si); 18.91 (C, $(CH_3)_3\underline{C}$-Si); -5.53 (CH_3,$(CH_3)_2$Si).
16. **4**. ^{1}H-NMR (250 MHz, $CDCl_3$): δ 7.43 (1H, bs, 6-H); 7.41-6.81 (13H, aromatic protons of trityl group); 4.38 (1H, m, 3'-H); 4.28 (2H, AB system broadened by long range coupling with 6-H, J_{AB}=13.8 Hz, 5-CH_2-O); 3.92 (1H, m, 4'-H); 3.79 (6H, s, -OCH_3); 3.39 (2H, further coupled AB system, 5'-H_2); 2.40 (1H, m, 2'-H); 2.19 (1H, m, 2''-H); 2.12 (1H, d, J=3.6 Hz, 3'-OH); 0.82 (9H, s, $(CH_3)_3$C-Si); 0.12 (6H, s, $(CH_3)_2$Si).
17. Mixture of two diasteroisomers; ^{31}P-NMR: δ 145.6 and 145.4 ppm.
18. Gait, M.J. *Oligonucleotide Synthesis A practical approach; IRL Press, Oxford* **1984.**
19. Oligonucleotide syntheses were conducted on a Beckman 200A synthesizer, using the recommended cycles and reagents, except that in the deprotection step a less concentrated DCA solution (2% in CH_2Cl_2) was used.
20. Phase sensitive NOESY (300 msec mixing time) of the hmU containing oligomer was performed on a GN500 spectrometer with a sample containing 2 mg DNA dissolved in 100 mM NaCl and 0.01 mM EDTA at pH 6.55 in D_2O at 25°C. Chemical shifts are referenced to TMSP-d_4.
21. Wüthrich, K. *NMR of Proteins and Nucleic Acids; John Wiley & Sons, New York* **1986**.